Summary

The initiation of an effective immune response depends on the proper development and optimal activation of the primary coordinators of the immune response, the dendritic cells (DC). In an immature state, DC are well equipped to take up and process antigens. Under normal circumstances, antigens taken up by DC will be self antigens, or antigens from harmless bacteria, in which case DC will not become activated and will not activate effector cells of the adaptive immune system, i.e. the T- and B cells. When DC take up foreign, or altered-self antigens in the presence of activating "danger" signals, they will mature which in turn leads to altered expression of chemokine receptors, resulting in subsequent migration to the draining lymph nodes, coinciding with upregulation of co-stimulatory molecules like CD80 and CD86 and increased antigen-presenting capacities. Fully matured DC can prime and activate specific T cells within the lymph nodes. The activated T cells are then prepared to eradicate cells that have become infected or express altered-self antigens. In case of cancer, tumor cells that are not eradicated by the immune system often develop strategies to suppress or "fool" the immune response, e.g. by secretion of suppressive cytokines like VEGF, IL-10, IL-6 and TGFβ or by down-regulation of MHC class I molecules or immunogenic tumor antigens. Immunotherapeutic strategies aim to alert and prime the immune system to fight cancer. DC are often used in immunotherapy, as they can present tumor-associated antigens (TAA) and thereby specifically activate TAA-specific T cells both for a rapid killer-response, as well as a memory response. DC-based therapies can consist of vaccination with *in vitro* cultured autologous (from the patient itself) or allogeneic (from a donor or cell line) DC, which are generated to present tumor antigens. Another way is to directly target DC *in vivo* to activate them and to load them with TAA, for instance by targeted therapies using adenoviral vectors. In order to make optimal use of DC for therapy purposes or to improve therapy strategies, it is important to know which factors influence DC development and functions.

ATP-binding cassette (ABC) transporters were first characterized for their role in the generation of chemotherapy-induced multidrug resistance (MDR). Over the years, it has become apparent that these transporters are also involved in protection of vital organs like the kidneys, the brain and the liver from endogenous and exogenous toxic compounds. More recently, ABC transporters were described to be expressed on cells of the immune system and several of them were shown to transport (pro-)inflammatory mediators like prostaglandins and leukotrienes. Chemotherapeutic agents themselves, although highly toxic when administered systemically and leading to immune ablation, were also found to act immunostimulatory when applied locally at lower doses.

For this thesis we had three main aims:

i)   Clarify the role(s) of ABC transporters in DC physiology and function.
ii)   Help delineate effects of cytotoxic drugs on DC and immune functions.
iii) Provide clues for augmenting DC functions in immuno-suppressed cancer patients.

Chapter 1 gives an introduction of the immune system and the role of DC therein, the use of DC for immunotherapy, the role of ABC transporters in chemotherapy resistance, their drug- and physiological substrates and their expression patterns on immune cells.

In **chapter 2** the importance of P-gp (ABCB1) and MRP1 (ABCC1) activity for the differentiation of DC precursor cells into functional immature DC is described. Whereas inhibition of P-gp activity had no effect on DC differentiation, inhibition of MRP1 activity during DC differentiation from blood monocytes or during interstitial DC (IDC) and Langerhans cell (LC) differentiation from MUTZ3 progenitor cells led to poor differentiation. This block

in DC differentiation was revealed by reduced expression levels of typical immature DC/LC markers like CD1a, CD1c, Langerin, but also co-stimulatory markers like CD86 and CD40 and the antigen-presenting molecule MHC II. In addition, MRP1-inhibited DC secreted lower levels of the pro-inflammatory cytokine IL-12 and MRP1-inhibited MUTZ3-LC displayed a reduced capacity to stimulate T cell proliferation. Beside that, we observed co-localization of MRP1 and the DC marker CD1a on *in vitro* cultured DC and found that the lipid CD1a ligands sulfatide and ganglioside GM1 could be transported by MRP1. As these newly identified MRP1 substrates and the previously identified leukotriene (LT) substrates LTC_4 and LTD_4 were not able to restore DC differentiation in the presence of MRP1 inhibition, the MRP1 substrate(s) required for efficient DC differentiation remains to be identified. Based on these data we conclude that active MRP1 is important for optimal differentiation of DC.

In **chapter 3** we report that human skin LC and IDC, as well as $CD34^+$ DC precursors from the blood, express BCRP (ABCG2) and that introduction of functional BCRP in MUTZ3 progenitor cells leads to faster differentiation kinetics and improved differentiation. Beside the acceleration of differentiation, which was confirmed on the signal transduction level by increased early RelB expression, a skewing towards a LC phenotype was observed. Even in the presence of the IDC-inducing cytokine IL-4 in the culture medium, these cells developed into cells with typical LC characteristics like CD1a^{hi} and Langerin expression. The emergence of these LC-markers appeared independent of Notch receptor signaling and was dependent on endogenously produced TGFβ. In conclusion, induction or introduction of BCRP in $CD34^+$ DC precursor cells could be a means to accelerate and enhance LC differentiation.

Since ABC transporters are known to transport cytostatic drugs, we looked whether the prominent drug-substrates mitoxantrone and doxorubicin (which are both cytostatic anthraquinone-derivatives) could somehow influence DC development. In **chapter 4** we studied the effects of mitoxantrone and doxorubicin on DC differentiation. While performing a cytotoxicity assay on MUTZ3 progenitor cells, we observed changes in cell morphology. Phenotypic analysis revealed that upon high-dose drug administration the cells started to differentiate into LC-like cells. Addition of these cytostatic anthraquinone-derivatives to MUTZ3-IDC or –LC cultures revealed a stimulatory effect on differentiation. Optimization of the culture protocol (focusing on mitoxantrone) provided us with mitox-DC which could be cultured into phenotypically mature IDC within 3-4 days, whereas conventionally cultured IDC take 7-9 days to develop. The mitox-DC were as capable as the day 7-9 control DC in migrating towards the lymph node homing chemokines CCL19 and CCL21, stimulating T cell proliferation and priming functional, TAA-specific CTL. We conclude that this new culture method, based on the adjuvant effect of low-dose mitoxantrone (or related drugs), could be a time- and cost-effective way to culture functional mature DC for therapeutic purposes.

In **chapter 5** we aimed to examine whether drug-selection of DC precursor cells (MUTZ3) with the ABC-transporter substrate doxorubicin would result in induced ABC transporter expression and subsequent effects on DC development. Though no changes in ABC transporter protein levels were detected, long-term doxorubicin exposure of MUTZ3 cells, transduced with the active domain of human telomerase in order to increase their lifespan (hTERT-MUTZ3), negatively affected the capacity of these cells to differentiate into LC. Dox-selected hTERT-MUTZ3 cells rapidly and completely lost the ability to differentiate. Fortunately, by deprivation of the selection drug for 3 months, the DC-differentiation capacity of the cells was restored. Dox-selection resulted in loss of expression of the stem cell factor receptor (SCF-R) on $CD34^+$ DC precursor cells. SCF-R expression returned upon removal of the drug, coinciding with the regained capacity of the cells to differentiate to DC. These data warn against immune suppression caused by insufficiently long cytostatic drug-free intervals, as specific DC

precursors may need time to recover from protracted chemotherapy treatment and re-emerge among the circulating CD34^{+} haematopoietic stem and precursor cells.

It had previously been reported that the ABC transporters P-gp and MRP1 play a role in DC migration. In **chapter 6** we demonstrate that MRP4 (ABCC4) is required for human DC migration. MRP4 expression was observed in human skin LC and IDC. Intradermal injection of the MRP4 antagonist sildenafil resulted in reduced skin DC migration. To confirm the MRP4 dependence of this migration, MUTZ3 progenitor cells were stably transduced with a short-hairpin RNA (shRNA) construct against MRP4, reducing MRP4 mRNA and protein expression. LC cultured from these MUTZ3-shMRP4 cells displayed reduced migration toward the lymph node-homing chemokines CCL19 and CCL21. In addition, intradermal administration of a DC-targeted adenovirus encoding shMRP4, led to a significant reduction in numbers of skin-emigrating DC, confirming a role for MRP4 in human DC migration.

In **chapter 7** we studied whether MRP4, which we found to be involved in human DC migration (chapter 6), was also important for the migration of murine DC and consequently for the initiation of immune responses in mice. Mrp4 expression levels were very low in murine skin and no difference was observed in DC migration from ear-skin of FVBwt mice or Mrp4 knockout mice. In addition, no altered immunological parameters or alterations in the induction of an immune response upon immunization were found between FVBwt and Mrp4 knockout or Mrp4/Mrp5 double knockout mice. We thus conclude that, unlike in man, there is no role for Mrp4 in DC migration in mice. This is inline with earlier reports on differences between ABC transporter expression and functions between these species.

Our observations that ABC transporters can regulate DC development (MRP1 and BCRP) and functions (MRP4) would suggest that increasing the expression of these transporters on DC precursor cells or DC could result in enhanced DC functionality. In **chapter 8** we aimed to identify a suitable, DC-specific targeted vector for DC-based immunotherapy, e.g. for the delivery of ABC transporter(-modulating) genes. Comparative analysis with different fiber-modified adenoviruses (Ad) showed that Ad5/3 selectively transduced mature human DC with high efficiency, whereas Ad5.RGD selectively enhanced transduction efficiency of immature cells as compared to wtAd5. Whereas Ad5.RGD did not prove to be more efficient in transducing human skin DC than the commonly used wtAd5, Ad5/3 *did* prove to be more efficient. Ad5/3 was shown to target human skin DC via CD80 and CD86, without compromising their T cell-stimulatory capacity. In skin single cell suspensions Ad5/3 was more specific than the previously identified DC-targeting vector Ad5/35, as it transduced less bystander cells. Also in human lymph node (LN) suspensions Ad5/3 specifically targeted mature DC subsets with high expression of the co-stimulatory markers CD80 and CD86. In conclusion, Ad5/3 is a suitable candidate for targeting mature human skin and LN DC for immunotherapeutic strategies. It could thus potentially be used to target MRP4-encoding genes to maturing DC, thereby putatively enhancing their migratory capacity.

In **chapter 9**, we discuss the importance of ABC transporters for the physiology of human DC. The consequences and implications of these observations for the treatment of cancer are discussed. Inhibition of ABC transporters to overcome multidrug resistance of tumor cells may negatively influence the potency of an anti-tumor immune response as well as render patients more susceptible for infections due to the negative effects this can have on the cells of the immune system. On the other hand, increasing ABC transporters expression on DC precursor cells or DC, e.g. through adenoviral transduction, could enhance DC development and functionality *in vivo*. This could be a new strategy to promote a pro-inflammatory anti-cancer response (since most physiological ABC

transporter substrates are pro-inflammatory) and in addition make these cells less susceptible for chemotherapy-induced cell death (as their presence on DC would allow the export of cytostatic drugs from the DC). The unraveling of the roles of ABC transporters on DC has been the first step in the direction of employing these transporters and their physiological functions to improve immunity. The next step would be to see whether increasing their expression levels on DC will actually augment DC functionality and thereby the effectiveness of the immune system against tumor cells.